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Genetics and Physiology of Colicin-tolerant Mutants of *Escherichia coli*

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A series of colicin-tolerant (*tol*) mutants of *Escherichia coli* K-12, which adsorbed colicins but were not killed by them, were isolated and studied genetically and physiologically. Three major classes of mutants were found: *tol II*, tolerant to colicins A, E1, E2, E3, and K; *tol III*, tolerant to A, E2, E3, and K; and *tol VIII*, tolerant to E1 only. The sites of *tol II* and *tol III* mutations mapped near the *gal* region (gene order: *tol-gal-bio*) and were cotransduced with *gal* by P1. In heterozygous diploids, tol^+ was dominant over *tol*; *tol II* and *tol III* gave full complementation. All the *tol* mutations that mapped near *gal* rendered the bacteria more fragile during growth and hypersensitive to deoxycholate and to ethylenediaminetetraacetic acid. The *tol VIII* mutation mapped between *str* and *his*. These mutants were extremely sensitive to deoxycholate and were also hypersensitive to methylene blue, acridines, and various other compounds. The sensitivity is attributed to increased uptake due to selective alteration of the permeability barrier. The colicin-tolerant mutations are interpreted as affecting some components of the cytoplasmic membrane which mediate between the adsorbed colicin molecules and the target sites of their biochemical effects in the bacterial cell.

The cytoplasmic membrane of a bacterium plays a major role in the functional organization of the bacterial cell. Not only is it the known or reputed site of many enzymatic activities—hydrogen transport, oxidative phosphorylation, lipopolysaccharide and murein biosynthesis (24)—but it has also been variously implicated in deoxyribonucleic acid (DNA) synthesis, protein synthesis, as well as in cell division and the concomitant orderly distribution of new copies of genetic material to daughter cells (9). Yet, the analytical study of the cytoplasmic membrane has barely started. By analogy with other problems of the functional organization of cells, a critical step in opening up the study of the cytoplasmic membrane may be a genetic approach, providing functional anomalies that can be traced to alterations of specific genes, of their products, and of the interactions of these products with other cellular components.

The work reported in this paper was initiated with the purpose of finding bacterial mutants that may be specifically altered in the functional organization of the cytoplasmic membrane. The system chosen was the resistance of *Escherichia coli* to colicins. The rationale for the approach is provided by recent work, especially by Nomura (18; Ann. Rev. Microbiol., *in press*), which suggests that the colicins exert their inhibitory role on specific cellular functions while remaining on the surface of the bacterial cell, and that at least the primary inhibitory functions are reversible by removal of colicin with proteolytic enzymes. The obvious candidate as the site of the inhibitory actions is the cytoplasmic membrane; the existence of mechanisms for generalization of the effects of a single colicin molecule to the membrane as a whole has been suggested to account for the one-hit kinetics of colicin action (13, 18).

Different colicins have different receptor sites on the cell wall, whose mutations render the bacteria resistant to specific colicins (3); for example, colicins E1, E2, E3 (and phage BF23) share identical or closely overlapping receptor sites. Different colicins also differ in their modes of action (18); colicins E1 and K arrest macromolecular syntheses and active transport, colicin E2 causes DNA breakdown, and colicin E3 inhibits protein synthesis.

For the purpose of the present work, mutants were sought that became tolerant (*tol*) to one or more colicins which they could still adsorb. The existence of such mutants was first discovered by Nomura (19). Among these mutants, one may expect to find "membrane" mutants, altered in the response to colicins because of alterations in specific membrane constituents. The initial search was made for mutants doubly tolerant to two

colicins, E1 and K, with different receptors but similar mode of action. This approach proved unnecessarily sophisticated because our own work, as well as reports from other laboratories (1, 19, 19*a*, 21), revealed that a substantial proportion of the colicin-resisting mutants were of the tolerant type, which adsorbs colicins but fails more or less completely to be inhibited by them.

The present paper describes some colicin-tolerant mutants of *E. coli* K-12 whose physiological characteristics indicate that these mutants have defects in some surface components which are recognizable independently of the tolerance to the colicin. Some of these mutants are similar to others described by Nomura (19), Clowes (1), and Reeves (21), and, in greater detail, by Nomura and Witten in the accompanying paper (19*a*).

MATERIALS AND METHODS

Media. The following media were used: LB (tryptone-yeast extract) broth and agar (14); LC (LB broth and agar plus 2.5 mM $CaCl_2$); λ broth and λ agar (tryptone, 10 g; NaCl, 2.5 g per liter); Penassay Broth (Difco); 63 minimal medium and agar [KH_2PO_4, 5.3 g; K_2HPO_4, 10.6 g; $(NH_4)_2SO_4$, 2 g; 1 N KOH, 3 ml; distilled water, 1 liter; $MgSO_4$, added to a final concentration of 1 mM]; Ozeki minimal medium and agar [K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; $(NH_4)_2SO_4$, 1 g; sodium citrate, 0.47 g; $MgSO_4$, 50 mg; distilled water, 1 liter]. Glucose or succinate was added at 0.4%; vitamin B_1 at 1 μg/ml; L-amino acids at 20 μg/ml; and streptomycin at a final concentration of 200 μg/ml. EMB-agar contained (per liter): tryptone, 8 g; yeast extract, 1 g; NaCl, 5 g; agar, 15 g; K_2HPO_4, 2 g; sugar, when present, 10 g; eosin Y, 300 mg; and methylene blue 50 mg. BTB-agar was the same as EMB but with 24 mg of bromothymol blue instead of eosin and methylene blue. Phosphate buffer was composed of KH_2PO_4, 3 g; Na_2HPO_4, 6 g; distilled water, 1 liter.

Soft-agar test of response to colicins. A colicinogenic strain was stabbed in an LB agar plate, which was then incubated overnight. The plates were sterilized by chloroform vapors, and a layer of melted top agar, inoculated with the strain to be tested, was poured on the plate surface (3). Resistant colonies were readily isolated from within the sterile halo formed by the action of the colicin on the sensitive strain.

Preparation of colicins. Colicinogenic bacteria were grown in 200 ml of LB broth to about 2×10^8 cells/ml; the cells were collected, suspended in 10 ml of saline, and irradiated in a petri dish for 60 sec (General Electric Germicidal lamp, 15-w, 50-cm distance). The cells were transferred to 400 ml of 63 minimal medium plus 10 mM $MgSO_4$ plus 0.5% tryptose (Difco), and incubated with shaking for 3 hr at 37 C. Cells were collected, suspended in 3 ml of buffer, and treated for 2 to 3 min in an MSE sonic oscillator. The sonic-treated suspension was centrifuged to remove whole cells and debris, and the supernatant fluid was frozen. The colicin titers were about 10^{12} killing particles/ml.

Mating procedures. The techniques of Jacob and Wollman (10) were followed. Platings on selective media were done after 2 hr of incubation at 37 C. The frequency of unselected markers among the recombinants was determined by transferring the recombinant colonies to plates of the same selective media and replica-plating onto appropriate media. For the test of colicin sensitivity or resistance, the replication was done on plates prepared by seeding with a colicinogenic strain in a soft-agar layer. After overnight growth, the soft layer was scraped off and the plates were sterilized with chloroform.

P1 transduction. P1 lysates were prepared by the method of Lennox (12). For P1 transduction, the procedure of Rothman (23) was followed.

β-Galactosidase assay. Free β-galactosidase was assayed by using *o*-nitrophenyl-β-galactoside (ONPG) as described by Revel, Luria, and Rotman (22). To test for ONPG hydrolysis by whole cells, the bacteria were grown in λ broth to an optical density (OD) value (420 mμ) of 0.4 to 0.5; 1 ml of culture in a spectrophotometric cuvette was mixed with 0.3 ml of 0.013 M ONPG in 0.25 M sodium phosphate buffer (*p*H 7.0), and the rate of formation of *o*-nitrophenol was measured at 420 mμ for 30 min.

F curing. For "curing" F′ strains by acridine orange, the method of Hirota (6) was followed.

Bacterial and phage strains. Bacterial strains used are listed in Table 1. Phage strains used are from the collection of this laboratory.

TABLE 1. *Bacterial strains*[a]

Strain	Relevant characteristics
Escherichia coli K-12 derivatives	
C600	F⁻ *thr⁻ leu⁻ thi⁻ lac⁻ (i⁺ z⁺ y⁻) str-s T1, T5-r*
W602	F⁻ *leu⁻ thi⁻ bio⁻ gal⁻ str-r*
W4032	Hfr Cavalli *pro⁻ met⁻ str-s T6-r*; polarity of transfer: *leu thr arg his trp gal*
PA309	F⁻ *thr⁻ leu⁻ thi⁻ trp⁻ his⁻ arg⁻ str-r*
AB1122-1	F⁻ *str-r*
LA348	F⁻ *str-r T6-r K-r*
W3101	F′ *str-s* (F-*suc⁺ gal⁺ bio⁺*)
P678	F⁻ *thr⁻ leu⁻ thi⁻ suc⁺*
P678 *suc⁻*	F⁻ *thr⁻ leu⁻ thi⁻ suc⁻*
Y20	F⁻ *thr⁻ leu⁻ thi⁻* (ColE$_1$-K30)
Other strains	
E. coli K235	(ColK)
E. coli CA38	(ColE3, ColI)
E. coli O6:H16,23	(ColA)
Salmonella typhimurium (*cys-36*)	(ColE2-P9)

[a] Symbols: *thr* = threonine; *leu* = leucine; *thi* = thiamine; *trp* = tryptophan; *his* = histidine; *arg* = arginine; *pro* = proline; *met* = methionine; *str* = streptomycin; *lac* = lactose; *gal* = galactose; *suc* = succinate; *bio* = biotin; T1, T5, T6 = phages T1, T5, T6; K = colicin K; r = resistance; s = sensitivity; Col = colicinogenic. Most bacterial strains used were from the collection of this laboratory. Strain W3101 was received from E. L. Wollman.

RESULTS

Mutants resistant to colicins were isolated from *E. coli* K-12 strains C600, W4032, and W602 without mutagenic treatment. Selection was done either with single colicins (A, or E1, or K) or with colicins E1 and K simultaneously by using the soft-agar test and the appropriate colicinogenic strains (Table 1). After purification by restreaking, the mutants were tested for sensitivity to a series of colicins and phages. The mutants that were studied further are listed in Table 2. In addition to the classical mutants, resistant only to colicin A, or to colicin K and to phage T6, or to colicins E1, E2, E3, and to phage BF23 (note that this type of mutant is resistant also to colicin A), there were several classes of mutants with other patterns of resistance. Five classes were recognized and named as follows: *tol II*, tolerant for A, E1, E2, E3, and K; *tol IIa*, partially tolerant for the same colicins; *tol III*, tolerant for A, E2, E3, and K; *tol IIIa*, tolerant for A, E2, E3, and partially tolerant for K; and *tol VIII*, tolerant for colicin E1 only. [The designations of various classes of mutants are provisional names selected to agree with those used in the accompanying article by Nomura and Witten (19*a*). The roman numerals are used to designate specific patterns of colicin tolerance.] Many of these *tol* mutants appear to arise by point mutations, since they revert at rather high frequency to colicin sensitivity. Multiple tolerance reverts in a single step. Most of these mutant types have their counterparts among the mutants reported by Clowes (1), Reeves (21), Nomura (19), and Nomura and Witten (19*a*).

TABLE 2. *Patterns of colicin resistance of spontaneous mutants isolated from Escherichia coli K-12 strains*[a]

Strain	Class	Colicins					Phages	
		K	E1	A	E2	E3	BF23	T6
C600	*tol*$^+$	s	s	s	s	s	s	s
	tol II	*r*	*r*	r	r	r	s	s
	tol III	*r*	s	r	r	r	s	s
	tol IIIa	*pr*	s	r	r	r	s	s
	tol VIII[b]	s	*r*	s	s	s	s	s
	E-r BF23-r	s	*r*	r	r	r	r	s
	A-*r*	s	s	*r*	s	s	s	s
	T6-r K-r	*r*	s	s	s	s	s	r
W4032	*tol*$^+$	s	s	s	s	s	s	r
	tol II	*r*	*r*	r	r	r	s	r
	tol VIII[b]	s	*r*	s	s	s	s	r
W602	*tol*$^+$	s	s	s	s	s	s	s
	tol II	*r*	r	r	r	r	s	s
	tol IIa	*pr*	pr	pr-r	pr	pr	s	s
	tol III	*r*	s	r	r	r	s	s

[a] Symbols: s = sensitive; r = resistant; pr = partially resistant. Italicized symbols correspond to the colicins used for the selection of a given mutant. The strain used as producer of colicin E3 also produced colicin I (*see* Table 1). Distinction between the two colicins was readily made on plates, since colicin E3 produced a much larger inhibition zone than colicin I; I-s E3-r strains showed only the small zone of inhibition by colicin I. All the *tol* mutants were sensitive to colicin V (from *E. coli* K94), B (from *E. coli* CL139), H (from *E. coli* CL10), D (from *E. coli* CA23), and I (from C600 ColI-P9).

[b] By the soft-agar method, the *tol VIII* mutants showed a decreased sensitivity to colicins A and K.

The *tol* mutants were tested also for sensitivity to phages T2, T3, T4, T5, T6, T7, λ, and MS2. They showed the same patterns of sensitivity as their respective parent strains. Additional types with partial tolerance for some colicins have been found but have not yet been studied.

Adsorption of colicins. Selected mutant strains derived from C600 were tested for ability to adsorb colicins E1 and K. Representative results are shown in Table 3. The mutants of types *tol II*, *tol III*, *tol IIIa*, and *tol VIII* adsorbed colicins about as effectively as did the parent strains. (Mutants of type *tol IIa* were not tested because they were not available in any *str-s* strains, as needed for our colicin-adsorption test. No significant results could be obtained with colicin A because of poor adsorption in liquid media.) The classical resistant types did not adsorb colicin to any measurable extent.

Genetic analysis of the mutants. Bacterial matings and transduction with phage P1 were used to map the various mutant types and showed that the *tol II*, *tol IIa*, *tol III*, and *tol IIIa* mutant sites map in a narrow region near the *gal* genes, whereas *tol VIII* maps not far from the *his* region of the *E. coli* chromosome.

Mapping of tol VIII. Crosses between strains W4032 Hfr *tol VIII str-s* and PA309 F$^-$ *tol*$^+$ *str-r* were performed and recombinants were selected and tested by replica-plating for various nonselected markers, including tolerance to colicin E1. Results of two typical crosses with a single *tol VIII* mutant donor are presented in Table 4 and indicate for this *tol VIII* mutation a location between the *his* and *str* loci. A similar location was reported for analogous E1-tolerant mutants by Clowes (1).

Mapping of tol II, tol IIa, tol III, and tol IIIa. Preliminary mapping by crossing W4032 *tol II* with PA309 *tol*$^+$ indicated that the *tol II* site was probably linked to *gal*. Linkage between *gal* and a multiple-colicin-resistance site had been encountered previously by Anton (Ph.D. Thesis, University of Buenos Aires, 1963) and Gratia (4). P1 transduction experiments (Table 5) were performed with lysates grown on *gal*$^+$ *bio*$^+$ *tol* strains to transduce *gal*$^+$ to *gal*$^-$ *bio*$^-$ *tol*$^+$ recipients. The *gal*$^+$ transductants were tested for

TABLE 3. *Adsorption tests for colicins E1 and K*[a]

Expt 1			Expt 2			Expt 3		
Strain used for preadsorption	Colicin E1 concn[b]		Strain used for preadsorption	Colicin E1 concn[b]		Strain used for preadsorption	Colicin K concn[b]	
	1.4×10^{-3}	10^{-3}		10^{-3}	2.5×10^{-4}		10^{-3}	2.5×10^{-4}
C600 *tol*$^+$	328	428	C600 *tol*$^+$	385	730	C600 *tol*$^+$	222	236
C600 E-r BF23-r	18	47	C600 *tol II*	595	700	C600 *tol II*	232	248
C600 *tol II*	388	457	C600 *tol VIII*	643	714	C600 *tol III*	223	248
None	14	95	C600 E-r BF23-r	37	92	C600 *tol IIIa*	218	236
			None	30	59	C600 K-r T6-r	0	24
						None	4	45

[a] An 0.2-ml sample of a log-phase culture in LB broth (2×10^8 to 5×10^8 cells/ml) of the (streptomycin-sensitive) strain to be tested was mixed with 0.1-ml colicin dilutions and incubated at 37 C. After 20 to 30 min of incubation, an 0.2-ml sample containing 200 to 600 cells of a *str-r* colicin-sensitive indicator strain was added to the mixture, which was further incubated for 10 min. The mixture then received 2.5 ml of melted soft agar with 200 μg of streptomycin per ml and was poured on streptomycin-agar plates. The colonies of *str-r* indicator strain were counted after incubation at 37 C. For colicin E1 (experiments 1 and 2), the indicator strain used was LA348; for colicin K (experiment 3), the indicator was AB1122-1. Thus, the results of experiments 1 and 2 are expressed as numbers of *str-r* survivors after test with colicin E1; those of experiment 3, after test with colicin K.

[b] Inverse of dilution factor from stock. When no colicin was added, experiment 1 yielded 460 *str-r* colonies; experiment 2 yielded 730; experiment 3 yielded 240.

TABLE 4. *Mapping of tol VIII mutants in the cross Hfr W4032 pro$^-$ met$^-$ tol VIII × PA309 thr$^-$ leu$^-$ arg$^-$ his$^-$ trp$^-$ str-r tol$^+$*

Expt	Selected markers	No. of colonies scored	Per cent of unselected markers				
			thr$^+$ leu$^+$	*arg$^+$*	*his$^+$*	*trp$^+$*	*tol VIII*[a]
1	*thr$^+$ leu$^+$ str-r*	80		31.3	3.8	2.5	1.3
	arg$^+$ str-r	108	60		1.9	0.9	3.7
	his$^+$ str-r	106	50	30		27.4	17.0
	trp$^+$ str-r	103	71	39	34		4.9
			str-s	*tol VIII*[a]	*his$^+$*		*try$^+$*
2	*his$^+$ pro$^+$ met$^+$*	103	26.2	40.7			29.2
	trp$^+$ pro$^+$ met$^+$	103	23.3	34.0	44.6		

[a] Resistance to colicin E1.

tol and *bio*. Reverse transductions were done with lysates from *gal$^+$ bio$^+$ tol$^+$* strains and *gal$^-$ bio$^-$ tol* recipients. One isolate of each of the strains mentioned in Table 5 was mapped in this manner. The results indicate the order *tol* – *gal* – *bio* for all four mutations *tol II*, *tol IIa*, *tol III*, and *tol IIIa*. The data do not permit us to establish a relative order among the various *tol* mutant sites.

P1 lysates grown of one isolate of C600 *tol II* were used to transduce *gal$^+$* to W602 *tol II*, *tol IIa*, and *tol III* (one isolate each), and the *gal$^+$* transductants were analyzed. We found that *tol$^+$* (colicin-sensitive) recombinants were produced in the *tol III* × *tol II* and *tol IIa* × *tol II* tests, but not in the *tol II* × *tol II* (0/160 tested) cross.

Close linkage of several *tol* mutants with *gal* has been reported also by Reeves (21) and by Nomura and Witten (19*a*).

Dominance and complementation analysis. The linkage with the *gal* region made possible the testing of several *tol* mutants in diploid condition for dominance and complementation. The F-*gal* factor present in strain W3101 (F-*gal*) carries the *suc*, *gal*, *bio* loci and the λ attachment site. This F-*gal* was transferred to *tol II*, *tol IIa*, and *tol III* derivatives of W602, and *gal$^+$* colonies were selected on EMB-galactose-streptomycin plates. Most of these *gal$^+$* strains proved to be diploids *gal$^-$/gal$^+$* and sensitive to the colicins. Their *gal$^-$* segregants were again tolerant. Also, when these

TABLE 5. *Mapping of tol II, tol IIa, tol III, and tol IIIa mutations by P1 transduction*[a]

P1 grown on	Recipient	No. of *gal*$^+$ transductants analyzed	Per cent of unselected markers			
			K-r	*bio*$^+$	K-r *bio*$^+$	
					Observed	Expected
C600 *tol II*	W602 *gal*$^-$ *bio*$^-$ *tol*$^+$	95	52	40	18	20.8
C600 *tol III*	W602 *gal*$^-$ *bio*$^-$ *tol*$^+$	105	58	47	27.6	27.3
C600 *tol IIIa*	W602 *gal*$^-$ *bio*$^-$ *tol*$^+$	102	67	50	30.4	33.5
			K-s	*bio*$^+$	K-s *bio*$^+$	
					Observed	Expected
C600 *tol*$^+$	W602 *gal*$^-$ *bio*$^-$ *tol II*	30	56.8	70	40	39.8
C600 *tol*$^+$	W602 *gal*$^-$ *bio*$^-$ *tol IIa*	28	46.5	75	35.6	35
C600 *tol*$^+$	W602 *gal*$^-$ *bio*$^-$ *tol III*	112	62.5	55	33	34.4

[a] The *gal*$^+$ transductants from EMB plates were picked and purified by restreaking; their *bio*$^+$ and colicin resistance characteristics were then determined. Expected values were calculated from the assumption of a map order *tol - gal - bio* and of integration of markers into the genome of *gal*$^+$ transductants without interference. A representative sample of the K-sensitive recombinants were tested against the other relevant colicins and found to be sensitive to them as well.

colicin-sensitive diploid strains were tested for colicin sensitivity, the inhibition areas exhibited many little colonies which proved to be mostly *gal*$^-$ *tol* segregants along with *gal*$^+$ *tol* recombinants. The dominance of *tol*$^+$ has also been demonstrated by Nomura and Witten (19*a*) for several *tol* mutations in the *gal* region.

An F-*gal* factor incorporating the *tol II* gene was obtained from a C600 *tol II* (F-*gal*) strain by plating on colicin K, picking colicin-tolerant colonies, and testing them for their ability to transfer *gal*$^+$ at high frequency. A strain designated C600 *tol II* (F-*gal tol II*) was used as donor of F-*gal tol II* to various derivatives of W602 (Table 6). It appears that there is full complementation between *tol III* and *tol II*, but no complementation between two *tol II* mutants (of independent origin). The mutations *tol II* and *tol IIa*, which confer different levels of tolerance to the same colicins, give partial complementation, possibly of the intracistronic type. With another mutant similar to *tol IIa* but somewhat more resistant to the colicins, no sensitivity was found in a partial diploid carrying F-*gal tol II*.

All diploid strains shown in Table 6 segregate out *gal*$^-$ cells with the colicin sensitivity pattern of the endogenote and also *gal*$^+$ *tol* recombinants.

The complementation studies suggest that mutations *tol II*, *tol IIa*, and *tol III* involve at least two different cistrons.

Kinetics of killing of partially tolerant strains by colicins. It is known from Fredericq's (3) and Nomura's (19) work that the immunity of colicinogenic strains to colicin is not absolute since it can be overcome by large concentrations of colicins. As pointed out by Nomura (19), the need for high colicin levels might reflect either a reduced efficiency of colicin action at individual sites or a requirement for cooperative effects at many sites (as would be expected, for example, if immunity resulted only from failure of a "spreading action" needed to generalize the effects of a local stimulus). Using the partially tolerant mutants *tol IIa* and *tol IIIa*, we found it possible to make a choice between the two alternatives. Figure 1 presents survival curves of various bacterial strains as a function of the concentration of colicins E1 or K acting on them for 10 min. It is clear that the killing kinetics, both for sensitive and tolerant strains, are of the one-hit type. The differences in slope of the killing curves correspond to factors of about 10 to 15 for colicin

TABLE 6. *Properties of strains diploid for tol genes*

Diploid	Sensitivity to colicins E1 and K	Sensitivity to DOC[a]
W602 *gal*$^-$ *tol*$^+$ (F-*gal*$^+$ *tol II*)	+	−
W602 *gal*$^-$ *tol II* (F-*gal*$^+$ *tol II*)	−	+
W602 *gal*$^-$ *tol IIa* (F-*gal*$^+$ *tol II*)	+	±
W602 *gal*$^-$ *tol IIa* (F-*gal*$^+$ *tol II*)	+	−

[a] *See* Table 9; ±, partial sensitivity (light growth at DOC concentrations of 0.5 and 1%).

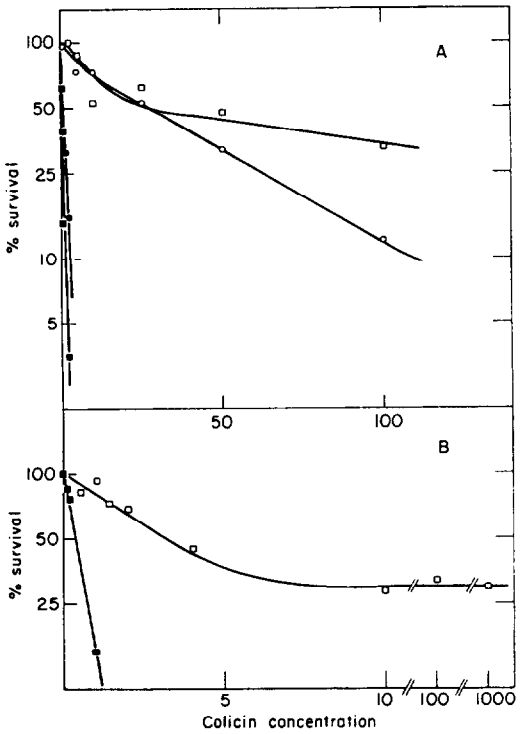

FIG. 1. *Kinetics of killing of tol IIa and tol IIIa mutants by colicins E1 and K. Mixtures containing 0.4 ml of bacterial cultures (2 × 10^8 to 4 × 10^8 cells/ml) and 0.1 ml of various colicin dilutions were incubated at 37 C. After 10 min, a 1:100 dilution was made in ice-cold broth. Samples were further diluted and plated for colony counts. (A) Colicin K; (B) colicin E1.* ■, *W602;* □, *W602 tol IIa;* ●, *C600 tol*$^+$*;* ○, *C600 tol IIIa. Note: in Fig. 1B the curve for strain W602 tol IIa reaches a plateau for colicin E1 concentrations about 20 times higher than that corresponding to 1 killing unit for strain W602 tol*$^+$*. If the plateau is reached because of saturation of receptors, this means that there are about 20 times as many receptors as there are molecules of colicin E1 in 1 killing unit.*

E1 and at least 20 for colicin K. We conclude that, in the partially tolerant mutants, there is a reduced average probability of a killing response to individual colicin molecules.

Physiological properties of tolerant mutants. A number of growth characteristics of mutants of types *tol II*, *tol IIa*, *tol III*, and *tol IIIa* were indicative of some abnormalities in the stability of cellular organization in these mutants. Further study revealed an unusual type of fragility which suggested a defect in some surface structure of the cells. At the same time, the similarity of our *tol VIII* mutants to the E1-tolerant mutants of Clowes (1) prompted us to reinvestigate the sensitivity of these mutants to dyes and led us to reinterpret the *tol VIII* defects in a way that also assumes a surface alteration.

Fragility of mutants tol II and tol III. All *tol II* and *tol III* mutants grew more slowly than did their parent strains. C600 *tol II* was studied in detail; it grew more slowly than C600 in a complex medium at all temperatures tested, but especially at 45 C (Fig. 2). Sucrose (0.5 M) gave only a slight protection at 42 C. In phosphate-glucose-ammonia minimal medium (with or without added citrate), C600 *tol II* grew very poorly; aggregates of partially lysed cells were visible in the medium.

As a measure of spontaneous cell disruption, the amount of β-galactosidase was determined in the supernatant fluid of cultures grown in a complex medium containing 5 × 10^{-4} M isopropyl-β-D-thiogalactoside (IPTG) as inducer of the *lac* operon. A much greater proportion of enzyme was released by C600 *tol II* than by C600 at all temperatures, but especially at 45 C (Table 7).

Advantage was taken of the fact that the *lac*$^-$ strain C600 is a β-galactosidase-positive (z^+), galactoside permease-negative (y^-) strain to differentiate *tol*$^+$ from *tol* mutants on agar plates in the following way. When 5 × 10^{-4} M IPTG was added to EMB-lactose-agar plates, the *tol* mutants were almost as dark red as *lac*$^+$ colonies, whereas the *tol*$^+$ strains gave pink colonies with or without IPTG. The *lac*$^+$ phenotype is interpreted as being due to the fact that the fragility of the *tol* cell causes a release into the surrounding medium of some galactosidase (which in z^+ y^- cells is induced by 5 × 10^{-4} M IPTG but not by 3 × 10^{-2} M lactose). Another difference was noted on minimal lactose-agar with IPTG added; after 48 hr of incubation at 37 C, C600 *tol* mutants formed large, rather mucoid colonies, whereas C600 *tol*$^+$ cells formed tiny colonies only, a difference probably due to release of β-galactosidase into the medium. Neither the release of β-galactosidase into liquid media nor the appearance of colonies on EMB-lactose-IPTG-agar was significantly altered by the addition of 0.5 M sucrose. Even when plated on EMB-agar without sugar, mutants of the *tol II*, *tol III*, and *tol IIIa* types from C600 were of a darker color than C600 *tol*$^+$ colonies.

The *tol II*, *tol IIa*, and *tol III* mutants derived from W602, which is *lac*$^+$, could also be distinguished from W600 *tol*$^+$ by their smaller size and darker color on EMB-agar without added sugars. The classical colicin-resistant mutants were indistinguishable from sensitive strains in any of these tests.

The colonial appearance of these *tol* mutants

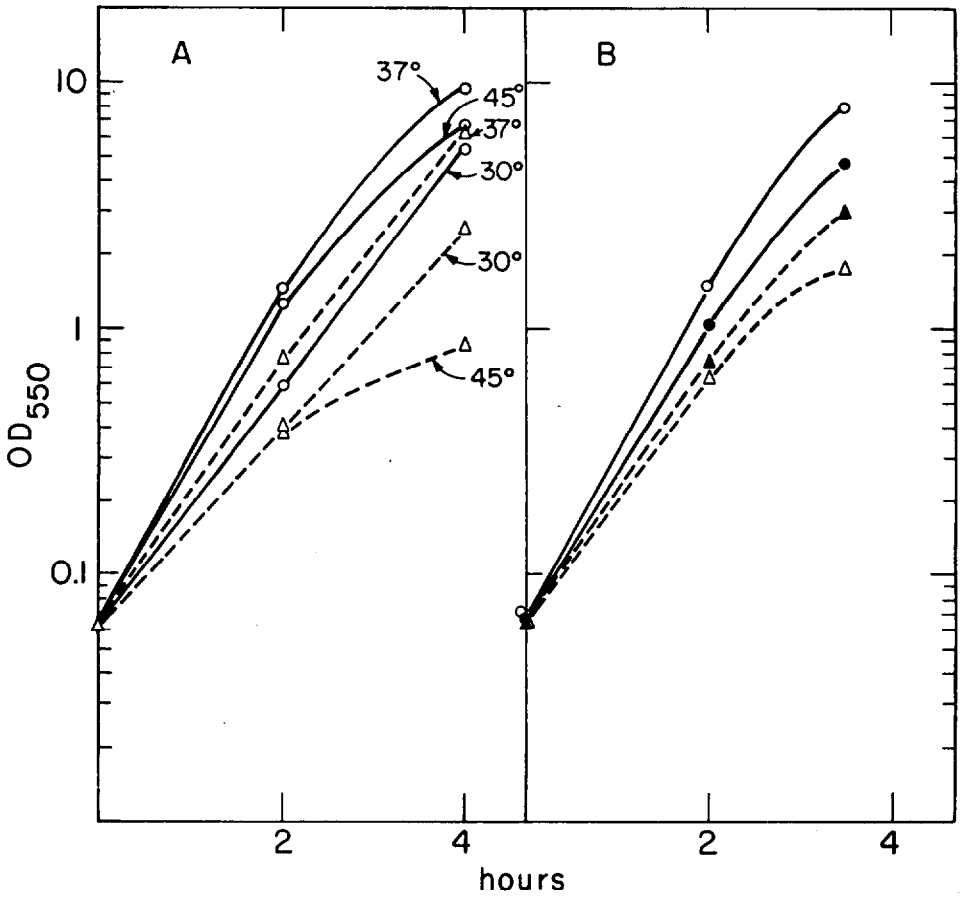

FIG. 2. *Growth of C600 tol⁺ and C600 tol II at various temperatures. (A) Overnight cultures were inoculated into λ broth plus yeast extract (0.1%) and IPTG (5 × 10⁻⁴ M). When an OD (550 mμ) value of 0.7 was reached, the cells were collected by centrifugation, resuspended, diluted in the same medium (prewarmed at the desired temperatures), and incubated with shaking. Samples were withdrawn at intervals for OD readings and β-galactosidase assay. After 2 hr, the cultures were diluted 1:5 with prewarmed medium; the 4-hr readings were corrected accordingly. (B) Effect of sucrose on growth at 42 C. Same conditions as in A, except that parallel cultures were incubated in media with or without 0.5 M sucrose. The bacteria for the sucrose cultures had been pregrown in sucrose medium. ○, C600 tol⁺; △, C600 tol II; ●, C600 tol⁺, sucrose; ▲, C600 tol II, sucrose.*

makes it possible to start experimental cultures from single mutant colonies, thereby avoiding accumulation of revertant types.

Dye sensitivity of tol VIII mutants. The *tol VIII* mutants did not show the increased fragility exhibited by the other *tol* mutants described above. Instead, as expected from the report by Clowes (1), *tol VIII* mutants proved to be hypersensitive to a series of dyes (Table 8). Experiments on the binding of methylene blue, acridine orange, and acriflavine were done in collaboration with R. Arditti, and revealed a much higher rate of binding by *tol VIII* than by *tol⁺* cells (Fig. 3). The binding of methylene blue was not eliminated but rather increased by KCN (Fig. 3A), an indication of a passive reaction.

The sensitivity of *tol VIII* mutants to acridine dyes was also tested by the effect of acridine orange on mutants carrying an F-*lac* factor. An F-*lac* factor was transferred to C600 *tol⁺* and C600 *tol VIII*, which are *lac⁻*. When the resulting *lac⁺* strains were treated with acridine orange, comparable curing effects were obtained with 2 and 5 μg/ml for C600 *tol VIII* F-*lac* and with 40 to 60 μg/ml for C600 *tol⁺*(F-*lac*). These findings are consistent with the hypothesis that *tol VIII* mutants have an increased permeability to dyes. Such increased permeability

of *tol VIII* mutants to dyes is not an instance of a generalized permeability for all substrates. For example, the rate of ONPG hydrolysis by whole cells of C600 *tol*$^+$ and C600 *tol VIII* is the same. Since in these z^+ y^- bacteria the rate of ONPG hydrolysis is limited by the rate of ONPG entry, an increased permeability should increase the rate of hydrolysis. Also, the *tol VIII* mutants are as resistant to actinomycin as is the parent strain.

Clowes (1) and Clowes and Moody (2) suggested that the sensitivity of E1-tolerant mutants to oxidation-reduction dyes is due to inability to reduce the dyes, possibly because of a defect in the succinate dehydrogenase. The *tol VIII* mutants described here grow on succinate as sole carbon source at rates not significantly different from those of their parent strains. A succinate dehydrogenase mutant (P678 *suc*$^-$; 7), which does not grow aerobically on succinate as sole carbon source, proved fully sensitive to colicin E1 and as resistant to methylene blue (and to deoxycholate; *see* below) as its *suc*$^+$ parent strain.

A study of reduction of triphenyl tetrazolium chloride (TTC) revealed further aspects of the dye sensitivity of *tol VIII* mutants. Reduction of TTC by intact cells leads to a precipitation of the red formazan, revealed by the color of a cell suspension or microscopically by the appearance of large red intracellular granules (11). Various concentrations of TTC were added to growing aerated cultures of C600 *tol*$^+$ and C600 *tol VIII*, aeration was stopped, and incubation was continued at 37 C for 15 to 30 min. At 0.005% TTC, *tol VIII* cells reduced the dye, but *tol*$^+$ cells did not; at 0.05% TTC, both *tol*$^+$ and *tol VIII* gave a positive red reaction; at 0.1% TTC, the *tol*$^+$ cells reduced the dye more effectively than did the *tol VIII* mutants; and at 0.5 or 1%, neither cell type could reduce TTC. We interpret these results as indicating that the mutant, being more permeable to TTC, can give a greater TTC reduction at lower concentration but is more effectively inhibited by concentrations that do not inhibit the *tol*$^+$ cells; the latter are themselves subject to inhibition by still higher levels of the dye.

In conclusion, all behavior of *tol VIII* mutants towards dyes appears to be better explainable by an enhanced penetrability by the dyes rather than by any specific disturbances in metabolic processes.

TABLE 7. *Release of β-galactosidase during growth of C600 tol*$^+$ *and C600 tol II at different temperatures*[a]

Time	Strain	Free β-galactosidase (% of total)		
		30 C	37 C	45 C
hr				
0	C600 *tol*$^+$		0.28	
	C600 *tol II*		1.1	
2	C600 *tol*$^+$	0.39	0.08	0.19
	C600 *tol II*	3.1	1.4	5.6
4	C600 *tol*$^+$	0.06	0.05	0.4
	C600 *tol II*	0.6	1.5	16.1

[a] The conditions of growth correspond to those described in Fig. 1.

TABLE 8. *Sensitivity of tol VIII mutants to dyes*[a]

Bacterial strains	Methylene blue (μg/ml in λ broth)					
	100	25	10	5	1	0
C600 *tol*$^+$, W4032 *tol*$^+$	+	+	+	+	+	+
C600 *tol VIII*	−	−	−	−	±	+
W4032 *tol VIII*	−	−	−	−	+	+
	Acridine orange (μg/ml in λ broth)			
	100	50	10	5
C600 *tol*$^+$, W4032 *tol*$^+$	+	+	+	+
C600 *tol VIII*, W4032 *tol VIII*	−	−	+	+
	Acriflavine (μg/ml in λ broth)			
	25	10	5	1
C600 *tol*$^+$, W4032 *tol*$^+$	−	+	+	+
C600 *tol VIII*, W4032 *tol VIII*	−	−	−	+
	Eosin Y (mg/ml in λ broth)			
	1	0.5	0.25	0.1
C600 *tol*$^+$	±	+	+	+
C600 *tol VIII*	−	±	±	+
	Triphenyl tetrazolium chloride (mg/ml in Penassay Broth)					
	5	1	0.5	0.1	0.05	0
C600 *tol*$^+$	−	+	+	+	+	+
C600 *tol VIII*	−	−	−	−	±	+

[a] A 1:100 dilution of an overnight broth culture of the strain was made in 2 ml of the medium indicated and incubated at 37 C with shaking. Full growth (+), absence of visible growth (−), or light growth (±) was recorded after 4 hr of incubation.

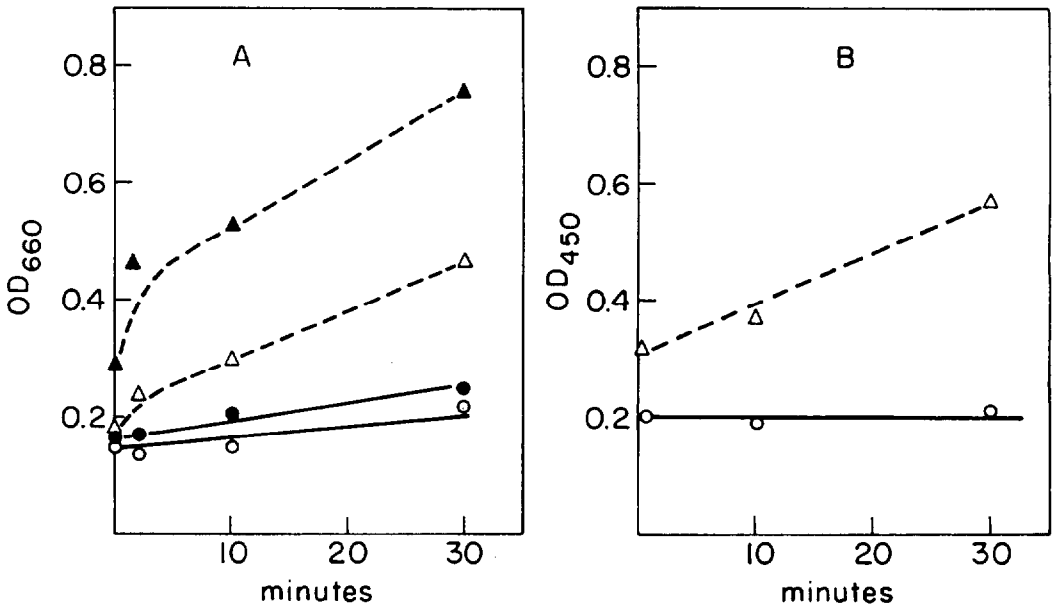

FIG. 3. *Binding of methylene blue and acridine orange to bacterial cells. (A) Cultures of C600 tol⁺ and C600 tol VIII were grown in minimal medium plus 0.4% glucose and 0.25% Casamino Acids to approximately 5 × 10⁸ cells per ml. Methylene blue was added at a final concentration of 10 μg/ml and the cultures were aerated at room temperature. At intervals, 2-ml samples were filtered through Millipore membranes (0.45-μ pore diameter) on filter pads. The membranes were transferred to 1 ml of buffer, the bacteria were resuspended, and 0.25 ml of a 25% solution of sodium dodecyl sulfate was added. The samples were incubated at 45 C until lysis was complete and then centrifuged to remove debris. The supernatant fluids were read in a Zeiss spectrophotometer at 660 mμ. KCN (10⁻³ M), when present, was added 3 min before methylene blue. (B) Same procedure, with acridine orange (100 μg/ml). Readings were done at 450 mμ. ○, C600 tol⁺; ●, C600 tol⁺ plus KCN; △, C600 tol VIII; ▲, C600 tol VIII plus KCN.*

Itikawa (8) reported that a group of E1-tolerant mutants similar to group VIII has increased sensitivity to 2,4-dinitrophenol and to chloramphenicol. This was found to be true also of the group VIII mutants described here.

A class of *E. coli* K-12 derivatives hypersensitive to basic dyes, apparently because of an enhanced uptake, has been described by Nakamura (16, 17) and Sugino (25). The corresponding gene maps near the *lac* region. These mutants are also more readily curable of an F factor by acridine orange.

Strain C600 derivatives carrying a Col E1 factor (E1-K30) and therefore immune to the colicin E1 do not show hypersensitivity to methylene blue. The *tol II* and *tol III* mutants do not show increased sensitivity to dyes, but are slightly more sensitive to penicillin.

Deoxycholate and EDTA sensitivity. When sodium deoxycholate (DOC), a mild surface-active substance, was added to growth media, all *tol* mutants proved to have heightened sensitivity (Table 9). This was especially true of *tol VIII* mutants, but all other *tol* mutants were more than 10 times more sensitive than *tol*⁺ cells. This effect of DOC was not significantly counteracted by addition of 0.5 M sucrose. Mutant *tol VIII* was very effectively killed in 30 min by treatment with 0.5% DOC (survival $<10^{-5}$) under nongrowing conditions, that is, in the absence of a carbon source and in the presence of chloramphenicol, with or without 0.5 M sucrose; under these conditions the *tol II* mutant was not more effectively killed than *tol*⁺.

The growth of *tol II*, *tol IIa*, and *tol III* mutants was completely inhibited by addition of 10^{-3} M ethylenediaminetetraacetate (EDTA) to LB medium, whereas *tol*⁺ or *tol VIII* mutants can grow in the presence of 5×10^{-3} M EDTA (Table 9). Attempts to counteract EDTA effects with various concentrations of divalent cations have given no clue to any specificity of action. On the other hand, strains C600 (ColE1-K30) and C600 (ColE2-P9), and the resistant (nonadsorbing) mutants, did not show this increased sensitivity to DOC or EDTA.

The actions of DOC and EDTA on bacterial surface layers are not well enough understood to

TABLE 9. *Sensitivity of tol mutants to DOC and EDTA*[a]

Bacterial strains	DOC (% in LB broth)						
	1	0.5	0.2	0.1	0.05	0.01	0
C600 *tol*$^+$; W4032 *tol*$^+$; W602 *tol*$^+$	+	+	+	+	+	+	+
C600 *tol II, tol III, tol IIIa*; W4032 *tol II*; W602 *tol II, tol IIa, tol III*	−	−	−	+	+	+	+
C600 *tol VIII*; W4032 *tol VIII*	−	−	−	−	−	+	+
	EDTA (molar concn in LB broth)						
	5×10^{-3}	2.5×10^{-3}	10^{-3}	5×10^{-4}	0		
C600 *tol*$^+$; W602 *tol*$^+$	+	+	+	+	+		
C600 *tol II, tol III, tol IIIa*; W602 *tol II, tol IIa, tol III*	−	−	−	+	+		
C600 *tol VIII*	+	+	+	+	+		

[a] Procedure as in Table 8.

permit specific interpretations, but the findings are in keeping with the conclusion that the *tol* mutants are altered in some surface components. DOC can presumably disrupt bonds between phospholipids and other membrane components. Increased sensitivity to DOC is found in *Salmonella typhimurium* mutants lacking the heptose as well as almost all other sugar components of the lipopolysaccharide core (M. J. Osborn and B. A. D. Stocker, *personal communications*), possibly because of a higher accessibility of lipoproteins to the reagent. The EDTA effect may bespeak the well-known (but poorly understood) role of divalent cations in membrane integrity and a greater sensitivity of some *tol* mutants to removal of such ions or a greater availability of them to the chelating agent.

Preliminary chemical studies on tol mutants. Through the courtesy of P. W. Robbins, some comparative analytical data were obtained on *tol*$^+$ and *tol* bacteria. The major phospholipid components of C600 *tol II* and W4032 *tol II* were analyzed by gas chromatography, and no differences were found from the analyses of the parent *tol*$^+$ strain. A set of *tol II* to *tol VIII* mutants of C600 was used to extract the lipopolysaccharide fraction, which after hydrolysis was tested for heptose. No differences were found between the mutants and C600 *tol*$^+$.

DISCUSSION

The attachment of a colicin to its cell-wall receptor triggers a series of events that affect a specific biochemical target, leading to cell death. On the one hand, colicins with apparenty common receptors, such as E1, E2, and E3, have different modes of action, hence different targets. On the other hand, some colicins with different receptors, such as E1 and K, apparently act on the same target.

Mutations to tolerance reduce the probability of successful action of a colicin on its specific target. Again, the specificity of the *tol* mutations cuts across the patterns of specificity of cell wall receptors and biochemical targets. *tol VIII* mutants are insensitive to E1 but not to the other E colicins nor to colicin K; the other *tol* mutants are insensitive to many colicins with diverse receptors and modes of actions. It seems reasonable to conclude that the *tol* mutations reveal another stage in the process of colicin action, presumably one that involves cellular components which mediate between the receptor-bound colicin molecules and the enzymatic constituents of the biochemical targets. The most likely candidates for the roles of mediators are components of the cytoplasmic membrane.

The present study of tolerant mutants has revealed an association between tolerance and alterations of certain cellular properties related to membrane functions. Thus, the *tol II* and *tol III* groups of mutants exhibit a definite cellular fragility and an increased sensitivity to DOC and to EDTA. The *tol VIII* mutants, besides being extremely sensitive to DOC, are abnormally permeable to a series of dyes. These observations are readily understood by postulating that the membrane components which mediate between the colicins and their ultimate biochemical targets also play roles in determining other membrane properties: stability, permeability, and sensitivity to reagents that affect the interactions among membrane components.

Our findings and their interpretation are in

line with the hypothesis first advanced by Nomura (18) that colicins inhibit cellular functions by interacting with specific constituents of the cytoplasmic membrane. The existence of mutants with temperature-dependent tolerance (19*a*) suggests that the relevant membrane components may be proteins.

An alternative explanation, which cannot be excluded at the present time, is that the mutations to tolerance alter the bacterial envelope in such a way as to activate mechanisms that destroy specific sets of colicins.

The recessive character of the *tol* mutations (at least those that map near the *gal* region) can be interpreted in a variety of ways. There might be a preferential incorporation of the wild-type product, or the mutant *tol* genes might make no utilizable products at all (in which case such products would have to be dispensable cell constituents), or a mosaic of tol^+ and *tol* gene products might be adequate for normal functions.

It is worth noting that tolerance to colicins differs from the immunity generated by colicinogenic factors. Immunity is epistatic to sensitivity, whereas tolerance is recessive. The specificity of immunity contrasts with the range of tolerance conferred by some of the *tol* mutations. Also, colicinogenic bacteria do not exhibit the sensitivities to methylene blue or DOC found in tolerant mutants.

Yet, it is reasonable to think that tolerance and immunity to colicins alter some cellular components that play specific roles in membrane organization. Some recent findings are relevant in this connection. Puig et al. (20; *personal communication*) described in *E. coli*, as well as in other bacteria, pleiotropic mutants that affect the function of several enzymes involved in anaerobic catabolism: formic hydrogenlyase, nitrate reductase, as well as thiosulfate and tetrathionate reductases. In *E. coli* some of these mutations map near the *gal* region, probably between *gal* and *bio*, and others map near the *mtl* (mannitol) region. The mutations appear to affect not the production of the relevant enzymes, but their assembly in functional form into a particulate element, which can be reconstituted by in vitro complementation between extracts of mutant strains. This situation suggests the existence of specific assembling substances in the cytoplasmic membrane of bacterial cells and recalls the apparent role of structural protein in the functional organization of mitochondria (5, 15). (A set of *tol* mutants from the present work was tested for ability to reduce nitrates and for gas production from glucose. All behaved like their parent strains.)

It is tempting to believe that the bacterial membrane contains a number of such substances, which act in the coordination of complex enzymatic processes such as those affected by various colicins and whose mutations may confer specific tolerance. The identification of these substances, their functions, their interactions among themselves and with enzymes and other components of the membrane should prove a difficult but rewarding task.

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LITERATURE CITED

1. CLOWES, R. C. 1965. Transmission and elimination of colicin factors and some aspects of immunity to colicin E_1 in *Escherichia coli*. Zentr. Bakteriol. Parasitenk. Abt. I Orig. **196**:152–160.
2. CLOWES, R. C., AND E. E. M. MOODY. 1965. Mutational immunity to a colicin. Proc. Soc. Gen. Microbiol., 43rd Gen. Meeting, p. xi–xii.
3. FREDERICQ, P. 1958. Colicins and colicinogenic factors. Symp. Soc. Exptl. Biol. **12**:104–122.
4. GRATIA, J. P. 1964. Résistance à la colicine B chez *E. coli*. Rélations de spécificité entre colicines B, I, et V et phage T1. Ann. Inst. Pasteur **107**:132–151.
5. GREEN, D. E., AND O. HECHTER. 1965. Assembly of membrane subunits. Proc. Natl. Acad. Sci. U.S. **53**:318–325.
6. HIROTA, Y. 1960. The effect of acridine dyes on mating type factors in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. **46**:57–64.
7. HIRSCH, C. A. M. RASMINSKY, B. D. DAVIS, AND E. C. LIN. 1963. A fumarate reductase in *E. coli* distinct from succinate dehydrogenase. J. Biol. Chem. **238**:3770–3774.
8. ITIKAWA, H. 1966. Changes in bacterial properties accompanied by the resistance to colicine E1 in *Escherichia coli* K12. Bot. Mag. (Tokyo) **79**:660–664.
9. JACOB, F., A. RYTER, AND F. CUZIN. 1966. On the association between DNA and membrane in bacteria. Proc. Roy. Soc. (London) Ser. B **164**:267–278.
10. JACOB, F., AND E. L. WOLLMAN. 1961. Sexuality and the genetics of bacteria. Academic Press, Inc., New York.
11. LEDERBERG, J. 1948. Detection of fermentative variants with tetrazolium. J. Bacteriol. **56**:695.
12. LENNOX, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
13. LURIA, S. E. 1964. On the mechanisms of action of colicins. Ann. Inst. Pasteur **107**:67–73.
14. LURIA, S. E., M. J. ADAMS, AND R. C. TING. 1960. Transduction of lactose utilizing ability among strains of *E. coli* and *S. dysenteriae*. Virology **12**:348–390.

15. MUNKRES, K. D., AND D. O. WOODWARD. 1966. On the genetics of enzyme locational specificity. Proc. Natl. Acad. Sci. U.S. **55**:1217–1224.
16. NAKAMURA, H. 1965. Gene-controlled resistance to acriflavine and other basic dyes in *Escherichia coli*. J. Bacteriol. **90**:8–14.
17. NAKAMURA, H. 1966. Acriflavine-binding capacity of *Escherichia coli* in relation to acriflavine sensitivity and metabolic activity. J. Bacteriol. **92**:1447–1452.
18. NOMURA, M. 1963. Mode of action of colicins. Cold Spring Harbor Symp. Quant. Biol. **28**: 315–324.
19. NOMURA, M. 1964. Mechanism of action of colicins. Proc. Natl. Acad. Sci. U.S. **52**:1514–1521.
19*a*. NOMURA, M., AND C. WITTEN. 1967. Interaction of colicins with bacterial cells. III. Colicin-tolerant mutations in *Escherichia coli*. J. Bacteriol. **94**:1093–1111.
20. PUIG, J., E. AZOULAY, AND F. PICHINOTY. 1967. Étude génétique d'une mutation à effet pleiotrope chez *Escherichia coli* K12. Compt. Rend. **264**:1507–1509.
21. REEVES, P. 1966. Mutants resistant to colicin CA42-E_2: cross resistance and genetic mapping of a special class of mutants. Australian J. Exptl. Biol. Med. Sci. **44**:301–316.
22. REVEL, H. R., S. E. LURIA, AND B. ROTMAN. 1961. Biosynthesis of β-D-galactosidase controlled by phage-carried genes. I. Induced β-D-galactosidase biosynthesis after transduction of gene z^+ by phage. Proc. Natl. Acad. Sci. U.S. **47**:1956–1967.
23. ROTHMAN, J. L. 1965. Transduction studies on the relation between prophage and host chromosome. J. Mol. Biol. **12**:892–910.
24. SALTON, M. R. J. 1964. The bacterial cell wall. Elsevier, Amsterdam.
25. SUGINO, Y. 1966. Mutants of *Escherichia coli* sensitive to methylene blue and acridines. Genet. Res. **7**:1–11.